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Creation and Optimization of an Optical Trapping Microfluidic Device for the Separation of Mock Forensic Sexual Assault Samples

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Abstract

Cell mixtures are often seen in forensic samples and commonly involve sexual assault cases where mixtures of sperm cells and vaginal epithelial cells are frequently encountered. This produces challenges in downstream analysis in the form of STR mixture profiles. The only method currently in use in crime laboratories for front-end sperm and epithelial cell separation is differential extraction. This method often results in STR mixture profiles due to carryover into both the male and female fractions and suffers from a wide range of efficiency depending on the laboratory or individual processing the sample. Optical trapping offers an alternative method for cell mixture separation by allowing cells to be individually selected and physically removed from a mixture. Previous studies exploring this method utilized an open droplet technique which had issues in the transferal of cells in a clean manner and displayed a high contamination potential. This research aimed to combine a microfluidic device with the optical trapping method to combat these issues. A microfluidic device was developed which allowed mixture samples to be passed through a micro-channel while target cells could be manipulated away and physically separated into a separate chamber downstream. Using this method, spermatozoa were trapped to produce a total of 13 single-source semen samples and 11 separated sperm cell:epithelial cell mixture samples. Separated cells were removed from the device and processed downstream using a standard forensic workflow. Resulting STR profiles demonstrated that this method produced minimal drop-in and female donor contributions from the trapped sperm fractions while producing full profiles from as few as 31 cells, with consistent full profiles observed with as few as 41 cells. Overall, this novel optical trapping microfluidic device allows for sperm cells to be successfully separated from a liquid mock sexual assault sample in approximately one hour and produces samples that can be immediately processed through a forensic workflow.

Keywords: Optical trapping, forensic science, cell separation, microfluidic device, mixture profile, spermatozoa, sexual assault

Introduction

Interpretation of STR profiles with multiple DNA contributors is an issue that is constantly faced in the field of forensic DNA analysis. There are certain situations in which these profiles can be easily sorted into two separate individuals, such as the case of a two-person mixture with a clear major and minor contributor. However, it is much more often that a mixture of two or more people that cannot be easily deciphered is encountered. The increase in sensitivity of STR technologies further intensifies this issue as previously unseen low-level contributors are more likely to be amplified and included in the profile. The hope for forensic science is to provide an unbiased, objective interpretation of criminal evidence. However, studies have shown that variabilities exist between laboratories and even individuals in the same laboratory in terms of how the same mixture profile should be interpreted [1]. These differences indicate that there could be bias in the process used. The Scientific Working Group on DNA Analysis Methods (SWGDAM) and other bodies have released regulations to guide analysts on which profile types are appropriate to analyze, but this still leaves many cases where interpretations cannot be made at all [2]. Much research is being done in an attempt to create methods that will eliminate this issue, both on the front end (cell separation) and the back end (more quantifiable genotype determination methods) [3-19].

One of the most common cell mixture types encountered in crime laboratories is a mixture of spermatozoa and epithelial cells, as is seen in sexual assault cases. In 2018 alone, the United States Department of Justice (DOJ) reported that there were 734,630 cases of rape and sexual assault which accounted for 11.5% of all violent crimes reported that year [20]. Statistically, this can be broken down into 2.7 cases of rape and sexual assault for every 1,000 people over the age of 12 years old, which was approximately double what it was in any of the

previous 4 years. The U.S. DOJ also reported that while the general trend since 1993 had been a decline of violent crimes, there has been an increase in recent years. In a separate report from the U.S. DOJ on recidivism rates of prisoners from 2005 to 2010, those who were convicted of a rape or sexual assault offense had a 20% likelihood to be arrested again within 1 year and a 60% likelihood to be arrested again within 5 years [21]. A 1997 report found that while rapists were less likely than other felons to be arrested for a new type of violent felony, they were more likely to be convicted of another rape [22]. Overall, the prevalence of these types of sexual crimes and recidivism rates of individuals convicted of them supports the idea that sexual assault kit backlogs will be a persistent problem that require improved methodologies to alleviate.

Cell Separation Methods Currently In Use

Currently, the method used in crime laboratories to separate cells from sexual assault samples is referred to as a differential DNA extraction. This method is only capable of separating spermatozoa and epithelial cell mixtures, as seen with sexual assault cases. The process relies on the knowledge that spermatozoa are generally less fragile than epithelial cells. Proteinase K and sodium dodecyl sulfate (SDS) are first applied to the cell mixture for a period of time to lyse the epithelial cells [23]. The sample is centrifuged to pellet the still intact spermatozoa while the genetic material of the non-sperm cells is free in the supernatant. The supernatant is removed and processed separately. To lyse the remaining spermatozoa, proteinase and dithiothreitol (DTT) are added which disrupts the disulfide bonds found in their acrosomal cap [23]. The process results in two separate fractions: a sperm fraction and a non-sperm fraction. The typical forensic laboratory workflow is then followed, with the goal being that the sperm fraction will contain only the profile of the male contributor who deposited the sperm.

While traditional differential extractions can be helpful in many cases and provide clearer information than non-separated mixtures, this process still has issues. Many sexual assault cases are not reported directly following the incident. It is known that the vaginal and digestive tracts degrade spermatozoa over time, leading to many cases where samples will contain degraded spermatozoa with weakened membranes. As a result, these cells can be weak enough to lyse alongside the epithelial cells and produce a mixture DNA profile. In addition, mixtures can also arise when genetic material in the supernatant is pulled into the pellet and processed alongside the sperm fraction, with the problem intensifying for samples with a low number of spermatozoa present [24]. Vuichard et al., (2011), found that the percentage of lost male DNA and the efficiency of cell separation using differential extractions ranged widely between laboratories using mock samples [25]. These issues present the idea that a more effective method for cell separation is needed, and preferably one that is capable of separating mixtures with varying cell types beyond sperm.

Probabilistic genotyping is another method that can be employed by crime laboratories in order to assist in mixture interpretation. In this method, statistical software is used to analyze a mixture profile and calculate which genotype determinations are most likely and/or calculate likelihood ratios [2]. Semi-continuous probabilistic genotyping software considers all possible genotype combinations for an STR profile and calculates likelihood ratios based on which STR alleles are present and the possibility of drop-out and drop-in STR alleles at each locus. However, they do not consider other variables such as peak height [26]. Continuous probabilistic genotyping methods consider the entire STR profile including which alleles are present, peak heights, stutter, etc. To make determinations, they use Markov Chain Monte Carlo (MCMC) modeling to perform simulations of the data under different circumstances in order to determine

the most likely circumstances of the target profile [26]. The program is then able to generate the resulting likelihood ratios for that profile. This method adds a quantifiable element to the process and improves consistency between laboratories employing the same software.

Ideally, the creation of front-end cell separation techniques would allow for single-source STR profiles to be produced that did not require the employment of probabilistic genotyping software. Additionally, there are some disadvantages that are currently faced when attempting to implement these programs in forensic laboratories. Many probabilistic genotyping programs are expensive. As with any other new technique employed in the lab, the software must also undergo internal validation to determine qualities such as precision, sensitivity, specificity, etc. [27]. This can be time-consuming while also removing an analyst from casework. Many fully-continuous programs are also proprietary, making them difficult to describe during courtroom testimonies.

Fluorescence Activated Cell Sorting

There are several methods currently in development to potentially address the issue of forensic cell mixtures. Fluorescence Activated Cell Sorting (FACS) combines the use of fluorescently tagging specific cells with the ability to separate or sort them after detection. Antibodies attached with fluorophores are used to target certain cell types in a mixture. Each cell is then passed in front of a detector which measures the absorbance and transmittance and the cells are sorted accordingly. Dean et al., (2015), found that this method was capable of separating two-person and four-person blood mixtures into clear separate contributors using fluorescently labeled human leukocyte antigen probes [5]. However, other experiments have proven this method to be less effective at sorting mixtures of different cell types. Research from Schoell et al., (1999), found that FACS improved the chances of producing a male-fraction STR profile in cases with low amounts of spermatozoa when compared to traditional differential

extractions, with 30% of samples producing a successful STR profile at a dilution of 160:1 compared to 0% using the traditional technique [6]. While this is an improvement to current methods, ideally, the success rate would be higher. In addition, Verdon et al., (2015), focused on separating cells from blood and saliva samples using antibodies anti-CD227 and anti-CD45 and found that the technique was largely unable to produce separate cell fractions [7]. For blood:saliva dilutions 1:5 and under, 100% of expected donor STR alleles were found. However, higher dilutions were unable to produce expected donor STR alleles, with only 57% of expected alleles produced from 1:10 and 1:50 dilutions, 25% from 1:100 dilutions, and 8% from 1:1000 dilutions. As a result, the probabilistic genotyping software STRmix was still required for deconvolution of many of the resulting STR profiles. It was noted that the antibody staining process is likely to result in the loss of cells, which could be an issue in forensic samples with low cell counts. Furthermore, cells with damaged membranes were noted to have an increase in non-specific fluorescence compared to fresh biological samples which would hinder the sorting of forensic samples in particular, as forensic samples are often older and dried [7].

Laser Capture Microdissection

Laser Capture Microdissection (LCM) is another cell separation technique currently being investigated for use in forensic contexts. LCM was originally developed for biomedical purposes by the National Cancer Institute [8]. Traditionally, the method is accomplished by placing a transparent thermoplastic film over the cell mixture and targeting cells of interest with an IR laser for a short period of time. The energy from the laser causes the film to attach to the cells, resulting in a removable film that contains only cells of interest. An alternative method involves the use of UV lasers to cut out particular cells and transport them into a collection device. This second method is generally preferred for forensic research as the lack of contact

with other materials minimizes the overall contamination risk of the sample. Previous studies have found mixed results from this method. Vandewoestyne et al., (2009), reported full STR profiles being developed from as few as 30 spermatozoa using this modified LCM method, with any fewer resulting in some allelic dropout [9]. However, Sanders et al., (2006), reported experiencing dropout with as many as 75 spermatozoa [10]. In addition, some researchers report issues with female DNA being present in the male profile while using this method [9,10].

While LCM is promising, there are a few issues that cause it to be currently unrealistic for implementation in forensic crime laboratories. LCM requires a significant amount of training for an analyst to possess enough skill to accurately use the technique. User error can cause cells to either not be released into the collection device, or can cause additional, unwanted cells to be collected as well. In addition, the agents used to fix cells for this technique are known to degrade DNA or are toxic to humans [11]. Furthermore, including this method would add hours to the normal sample workflow [12]. Unfortunately backlog is an issue that cannot be ignored in forensic laboratories, and as a result, time is a real consideration when incorporating new technologies.

Dielectrophoretic Trap Array

Dielectrophoretic Trap Array (DEPArray) is a separation technique which manipulates cells based on their fluorescence and morphology. DEPArray relies on the use of a nonuniform electric field to cause the movement of particles, trapping them in a designated position on a microdevice. Biological cells, in particular, are noted to have diverse dielectric properties which allows this method to be particularly effective for sorting cells with different morphologies [13]. In this method, cell-specific antibodies are tagged with fluorophores and bound to cells in a mixture. The mixture is then subjected to the DEPArray which moves and isolates each cell from

the mixture into an individual trap. Cell sorting software is used to analyze each cell individually, which are then moved and grouped using dielectrophoretic forces. Recent research has found this method to be quite sensitive and specific. Williamson et al., (2018), found that the DEPArray system was able to produce single source profiles in 96.2% of cases as compared to 32.1% of cases when using traditional differential extractions [14]. In addition, research from Fontana et al., (2017), found complete precision when separating epithelial and sperm cell mixtures found in mock forensic settings, in that sperm and epithelial cells were completely identified and separated correctly [15]. However, a large concern for the DEPArray technique is the amount of time required to process one single sample. While the device only requires approximately two hours of active handling from an analyst, it requires anywhere from 8 to 32 hours to actually process a single sample [16]. Considering that the technique can only be used for one sample at a time, this timeframe is neither ideal nor practical for many crime laboratories given the volume of cases they regularly receive.

Optical Trapping

The study described herein utilized optical trapping as a cell separation technique to solve the practical issues seen in previously discussed methods. Optical trapping utilizes a highly focused laser beam to trap and manipulate objects within a liquid medium [28]. Light has both a momentum and a direction of propagation, and when photons from the laser interact with a particle, the photons are scattered and the change in their momentum causes an equal and opposite momentum change for the object. In addition to this scattering force, lasers can be focused through a strong (high numerical aperture) lens to create a steep intensity profile, which in turn creates a large gradient force that can pull objects with a higher index of refraction (relative to the surrounding solution) towards the center of the beam. If the gradient force is able

to overcome the force from the scattering photons, the object is effectively trapped in the center of the beam and is able to be physically moved [28]. Optical trapping can be used in conjunction with both microscopes and cameras to better visualize manipulation on a cellular level [29].

Arthur Ashkin is credited for pioneering the field of optical trapping, starting in the 1970s with a series of published papers detailing theoretical work on the subject. His report of his success with the first optical trap was published in 1986 [30]. Ashkin's original experiments focused on manipulating inorganic particles and examining the effects of various laser intensities on those particles. He eventually moved on to living specimens, manipulating viruses, live bacteria, and erythrocytes in 1987. These experiments focused on damage seen to the cells during and following trapping. Notably, *E. coli* and *S. cerevisiae* were able to reproduce while actively in the trap over the course of five hours, indicating an absence of laser induced cell damage. In addition, erythrocytes and virus particles were shown to have no obvious morphological damage [31,32]. It was therefore deduced that the process of optical trapping was safe for the manipulation of biological cells.

Previous Optical Trapping Research at Virginia Commonwealth University

Previous work from Auka et al., (2019), focused on manipulating spermatozoa samples diluted in phosphate buffered saline (PBS) and bovine serum albumin (BSA) using optical trapping [17]. In this study, samples were placed onto glass coverslips (#1.5) in a droplet form and spermatozoa were gathered into a single point on the side of the droplet using optical trapping. Initial testing involved collecting the gathered fraction of the sample using a glass capillary which was then placed into a microcentrifuge tube for further analysis. This method resulted in low DNA yields, which were believed to be due to surface tension inside the capillary limiting the reagents' ability to reach the sperm cells. To combat this issue, the cover slip

collection method was created. This method involved placing the cells from the capillary onto a microscope cover slip to confirm the number of cells present before transferring the coverslip to a microcentrifuge tube to undergo further DNA analysis, including extraction, quantification, and STR profile interpretation. Samples containing various numbers of sperm cells were tested; approximately 50 spermatozoa were required to produce a full STR profile using the AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Applied Biosystems, Waltham, MA) [17]. Samples containing lower than 50 spermatozoa had increases in allelic drop-in and drop-out, which is commonly observed with low copy number samples. This method did suffer from contamination events, but they were heavily mitigated by increasing contamination precautions such as increasing the use of personal protective equipment and autoclaving microscope slides directly before use in subsequent experiments. Of two mock mixture samples (spermatozoa and epithelial cells) tested, 51 and 56 sperm cells were collected which produced single-source STR profiles, with 97% and 100% of expected alleles present, respectively [17].

Research has also been conducted on the feasibility of the use of optical trapping for the separation of blood samples. Specifically, venous blood samples were treated with an ammonium-chloride-potassium (ACK) lysis to destroy erythrocytes and were stored in PBS prior to optical trapping [18]. Ultimately, low DNA yields were initially obtained when using the same technique to verify number of cells prior to extraction as before. It was theorized that the leukocytes were lysing directly onto the glass coverslip and the resulting released DNA was preferentially binding to the silica composition of the coverslip. To combat this issue, leukocytes were isolated from the samples and injected directly into ATL lysis buffer (Qiagen, Oberkochen, Germany) before undergoing further DNA analysis. Of six samples, each containing exactly 10 leukocytes, two were able to generate full STR profiles with the remaining four resulting in

76.7%-95.3% of expected STR alleles observed. Samples with as few as six leukocytes were able to produce almost complete profiles (97.7% expected alleles) [18].

Further research by this group focused on optimizing the optical trapping methodology for forensic use. Spermatozoa were isolated and directly injected into ATL lysis buffer in the same manner as leukocyte samples to determine if this method was similarly viable. Findings were consistent with Auka et al., (2019), indicating that at least 50 spermatozoa were needed to produce a full STR profile for both liquid samples as well as reconstituted epithelial and spermatozoa mixtures [19]. The female contributor was observed in the resulting STR profiles from only two liquid samples, which was likely due to extra cells being inadvertently collected by the capillary. The amount of time required to trap 50 spermatozoa was notably only 20-30 minutes. Unfortunately, when reconstituted blood samples were tested using this method it was noted that leukocytes had a changed morphology that caused trapping to become more difficult [19]. DAPI staining of the leukocytes prior to trapping confirmed that the cells were intact, however, the DAPI stain caused the cells to be repelled by the laser and therefore unable to be isolated after staining [19].

Microfluidic Devices

Microfluidic devices, as the name suggests, are devices in which micrometer-sized channels are employed to manipulate liquids. The allure of microfluidic devices is their ability to be incorporated into existing technologies, as seen in a wide range of biomedical diagnostic and cell manipulation uses [33-35]. In forensic contexts in particular, microfluidic devices have been created for both serological testing for the identification of body fluids and DNA workflow purposes [36,37]. Most microdevices created for forensic DNA purposes only include one step in the workflow (extraction, purification, amplification, and separation), but there are laboratories

that have successfully included the entire process from swab to profile [38,39]. Cox et al., (2016), successfully created a microfluidic device composed of a sort of plastic called PMMA (polymethyl methacrylate) which could produce CE-ready STR profiles from a buccal swab cutting. The microfluidic device was able to produce profiles from a swab within 115 minutes using a system of valves and centrifugal force in combination with infrared-mediated PCR [40].

There have also been attempts to develop microdevices for the purposes of separating sperm:epithelial cell mixtures. Demirci et al., (2018), created a microdevice that isolated sperm cells using a unique oligosaccharide sequence which is involved in sperm-egg binding *in vivo*. Results were quickly achieved, however epithelial cells were not able to be completely flushed out and some still remained in the sperm fraction [38]. Other techniques involving separating sperm and epithelial cells based on size and morphology have been attempted, however, reports demonstrate their inability to control the free-floating DNA from epithelial cells that are damaged from the transition from swab to liquid [39,40]. Finally, some microdevices designed to separate cell fractions have incorporated other previously discussed techniques such as FACS and DEPArray, but these techniques largely still have the issues discussed before [44,45].

Use of Microfluidic Devices with Optical Trapping Techniques

The overall objective of this project was to build upon previous work using optical trapping through the addition of a microfluidic device to address several concerns. Previous studies using an open droplet technique with optical trapping found that contamination events and the collection of additional, unwanted cells were concerns [17,19]. The introduction of a microfluidic device offers a potential solution by allowing cells to be separated in a closed environment, thereby lowering the risk for contamination and potential sample loss due to manual transfer steps. In addition, channels could be added to a microdevice that would allow for

the physical separation of cell fractions, eliminating the risk of additional cells being present in the fraction due to the retrieval process as was seen when unwanted cells were collected via capillary using the open droplet technique.

Several goals in this project were designed to more fully explore the utility of a microfluidic device for optical cell trapping. Firstly, a microfluidic device architecture had to be designed that was able to manipulate the flow of cells in a medium to allow for optical trapping to occur. It was then necessary to test the effectiveness of the device based on its ability to separate and isolate samples using human cell types and cell mixtures. This was first addressed using semen samples, and later expanded to include sperm:epithelial cell mixtures. STR profiles resulting from isolated samples were then analyzed to determine the quality of the profiles, as well as determine the number of cells that were required to produce a full STR profile.

Methods

Sample Collection

All samples were collected in accordance with approved VCU IRB protocols (VCU HM20002931). Semen samples were collected in sterile containers and stored at -20° C. Vaginal epithelial cells were collected on sterile cotton swabs and stored at room temperature. One donation from one donor was used for the entirety of the project for each fluid type.

Sample Preparation

To resuspend vaginal epithelial cells, swab cuttings were added to 300 µL deionized H_2O and incubated for five minutes at room temperature. Swab cuttings were kept in the sample following the incubation. Semen samples were diluted twenty-fold using 4 mg/mL bovine serum

albumin (BSA) in ddH_2O. To create cell mixtures, equal volumes resuspended vaginal epithelial samples and 1:20 diluted semen samples were combined.

Optical Trapping Laser Setup

An AxioObserver D1 inverted microscope (Zeiss, Thornwood, NY) fixed to an air-floated 3' x 4' vibration isolation table was used alongside a motorized microscope stage that was controlled with a joystick. For trapping, an oil immersion 100x objective served as the optical tweezer focusing element for the 700 mW, 1064 nm continuous wave (CW) laser (CrystaLaser, Reno, NV) to be focused through. The laser was attenuated with an OD1 neutral density filter, power at the trap focus was measured at 25 mW, and aligned with appropriate optics into the back aperture of the microscope objective. The infrared (IR) laser trapped the cells one at a time in its center, and each trapped cell was moved to the extraction zone. Once a cell reached the extraction zone, it was released from the optical trap and cell collection continued.

Microfluidic Device Design, Fabrication, & Optimization

AutoCAD LT® 2018 software (Autodesk®, San Rafael, CA) was used to design a 5-layer microfluidic device consisting of polyethylene terephthalate, printer toner, and heat-sensitive adhesive. The design was exported to a VLS 3.50 software system and each layer was individually cut using a VersaLaser® CO_2 laser ablation instrument (Universal Laser Systems, Scottsdale, AZ). To ensure that the device could easily be used in conjunction with a microscope, negative space was incorporated into the bottom layers so that a plastic microscope coverslip could be attached. The layers and plastic microscope coverslip were then aligned and subsequently bonded together using a AL13Ps laminator (Apache Laminators, Humacao, PR). Several preliminary iterations/designs were tested in order to determine which worked best for

optical trapping purposes. Each design contained small ports connected by channels where samples are deposited and separated fractions can be retrieved (Figure 1).

Polystyrene beads (Bangs Laboratories, Fishers, IN) three micron wide were originally used in place of cells to test the device and determine which design functioned best. The beads were manipulated into channels on the device in a similar manner to cells. The number of beads that adhered to the surfaces of the chip were also monitored. The device was determined to work successfully when the flow of beads could be controlled in a manner that allowed for individual beads to be trapped and placed in an isolated area, with no beads randomly flowing into that area. Once a chip design was proven to successfully work as intended, the design was tested with human cells. Original tests addressed the separation of individual sperm cells from neat semen samples followed by testing using semen and vaginal epithelial cell mixtures. Small changes to sizing and angles of channels were made over time as needed. The initial and final designs of the microfluidic device are shown in Figure 1.

Cell Separation

To prime the device for samples, enough ddH_2O was flowed through the device to fill all channels and create pockets of water on the surface at ports A and B (Figure 2A). Kwik-Cast™ (World Precision Instruments, Sarasota, FL) was then added to port C to ensure it was sealed, as well as to the corners of the device to anchor them to the stage (Figure 2B). Once the Kwik-Cast™ had solidified, 2 µL of sample was added to either port A or port B depending on the orientation of the chip (Figure 2C). After approximately one minute, verification of cells flowing through the trapping area was confirmed microscopically with the 10X objective. The 100X oil immersion objective was then used in conjunction with the laser to capture individual sperm cells (Figure 2E). Cells caught in the focal point of the laser were transported to the extraction zone by

moving the mechanical stage relative to the fixed laser focus (Figure 2F, 2G). In order to determine whether or not the microfluidic device altered the number of sperm cells required to produce a full profile, the number of cells captured in each sample varied. After the target number of cells had been captured and moved to the extraction zone, the device was cleaned on the surface using sterile swabs covered in 20% bleach and 70% ethanol around the extraction area. It was then cut using sterile scissors to physically separate the extraction zone from the trapping area (Figure 2H). The device was then observed again as the extraction zone dried to ensure that the cells were still present. The device piece was allowed to dry, after which the extraction zone was completely excised from the device and placed in a 2.0mL microcentrifuge tube (Figure 2J). The sample was then stored at -20°C until extraction.

DNA Extraction

Samples were extracted using the QIAamp DNA Investigator Kit (Qiagen) according to the manufacturer's protocol "Isolation of Total DNA from Surface and Buccal Swabs". The protocol was modified to exclude the addition of carrier RNA at the lysis step. A volume of 20 µL of DTT was added alongside proteinase K in order to properly lyse the sperm cells. The device piece was exposed to all lysis reagents outlined in the protocol. To ensure all sample was removed from the device piece prior to adding sample to the silica column, the piece was placed in a spin basket (Promega, Madison, WI) and spun at 13,000 RPM for one minute. The spin basket and device piece were then discarded. DNA was eluted in a volume of 30 µL and stored at -20°C prior to quantification.

DNA Quantification

Samples were quantified using the Quantifiler Trio Kit (Applied Biosystems) on the ABI Prism 7500 Real-Time PCR System (Applied Biosystems) following the manufacturer's protocol modified for half volume reactions. In accordance with the protocol, standards were run in duplicate. A total yield was calculated for each sample by multiplying the quantity of DNA found in the small autosomal target by the elution volume (30 µL). In addition, a degradation index (DI) was calculated for each sample by dividing the concentration of the small DNA target by the concentration of the large DNA target.

STR Amplification

Samples were concentrated to 7.5 µL using a Savant DNA120 SpeedVac concentrator (ThermoFisher Scientific, Waltham, MA) at a low drying rate with no heat prior to amplification. The entirety of the sample was then added to the amplification reaction. Samples were amplified on the ProFlex PCR system (Applied Biosystems) using the PowerPlex® Fusion 5C kit (Promega) for half volume reactions. Thermocycling parameters were as follows: 96°C for 1 minute followed by 30 cycles of 94°C for 10 seconds, 59°C for 1 minute, and 72°C for 30 seconds, then 60°C for 45 minutes followed by a 4°C hold indefinitely.

Capillary Electrophoresis & STR Analysis

Following amplification, samples were separated using capillary electrophoresis. Each well of the 96-well plate contained 0.3 µL of WEN ILS 500, 9.7 µL of Hi-Di Formamide, and either 1 µL of sample or PowerPlex® Fusion 5C allelic ladder. The plate was heat denatured at 95°C for three minutes and then immediately placed in a freezer block that was kept at -20°C for 5-10 minutes. The plate was then run on an ABI Prism 3130 genetic analyzer (Applied Biosystems). Parameters were as follows: 3 kV injections for 5 seconds into 36 cm capillaries

containing POP-4 polymer (ThermoFisher Scientific). Resulting STR profiles were analyzed using GeneMapper® software version 4.1 with an analytical threshold of 50 RFU.

Statistical Analysis

Results were analyzed using several factors including total yield, percentage of expected alleles observed in the profile, number of drop-in alleles, number of alleles from the female contributor, and average peak height. A common approximation for the amount of DNA contained within a diploid human cell is 6.0 pg, therefore theoretical yields for each sample were calculated based on the knowledge that each sperm cell should contain approximately 3.0 pg of DNA as they are haploid [46,47]. This value was compared to the total yields calculated during quantification. In addition, the number of cells in each sample was compared to the percentage of expected alleles seen in each sample's STR profile, and to the average peak height for each sample. Average peak heights were calculated by averaging the heights of all called peaks present in each profile, where values for homozygous peak heights were assumed to consist of two alleles and therefore were divided by two. A linear regression was fit to determine the correlation between cell number and average peak height using R v4.0.2 (University of Auckland, New Zealand)

Alleles present that were not consistent with the expected profile of the semen donor that were consistent with the female contributor were assumed to be from the female contributor. Other alleles that matched neither donor were assumed to be "drop-in" alleles and were counted as such.

Results and Discussion

Microfluidic Device Design, Fabrication, & Optimization

Preliminary designs of the microfluidic device focused on the idea of using centrifugal force to remove cells from the trapping area once sample collection was complete. Earlier iterations had four separate channels with open ports to support this plan, as seen in Figure 1A. However, this design had several flaws that needed to be addressed. Not enough opposing force existed to prevent cells from dispersing in all four channels. This prevented the success of any cell separation as it was impossible to isolate select cells. In addition, it was also noted that cells would stick to the surface of the coverslip and the walls of the channel. This gave doubt to the ability of the initial device architecture to clear all cells out of the device once trapping was complete.

Several iterations of designs were tested before striking a balance that allowed trapping to occur. The design was ultimately modified to have three channels with a port that would be blocked to atmosphere in a manner that would allow cell flow to be controlled (Figure 1B, 2). Blocking port C using Kwik-Cast^{TM} after adding liquid medium but before adding sample prevented random cells from flowing into the channel and allowed the extraction zone to remain empty. It was determined that clearing the device entirely of unwanted cells using centrifugal force would not be feasible due to the previously mentioned issue of cells adhering to the coverslip. To combat this, a system was designed whereby the extraction zone could be physically cut from the device in order to secure the trapped cells after separation. This small device piece containing the extraction zone could then be exposed to the lysis steps during the extraction process and removed immediately prior to sample being added to the silica column for DNA purification. Changes made to the device once sample collection began were minimal and dealt with small changes to the channel width and length between the trapping area and extraction zone to ease the trapping procedure. The final device design is shown in Figure 1B.

The final series of cuts to separate the extraction zone from the device (Figure 2I) created a large source of variability for sample collection. The device was created with materials that were fragile enough to separate occasionally when twisted with the force required to perform the detailed series of cuts with scissors. As a result, it was sometimes the case that the device and coverslip would separate during this procedure. There were no direct impacts observed as considerable differences in DNA yields or completeness of DNA profiles between samples with intact and separated devices were not noted. However, alternate methods to perform this step should be explored in future projects to prevent possible contamination events and to ease processing.

In early samples, the two layers of the extraction zone were physically separated using sterile tweezers so that the extraction zone would be fully exposed to lysing agents during the extraction process. While these layers separated quite easily with initial samples, they were much more resistant with later samples and became impossible to separate. As a result, the final method included the entire extraction zone, without separation. This also did not appear to have an impact on the extraction process as determined by the lack of difference between total yields seen in samples prior and after this change.

Polystyrene Beads

Trapping was attempted on each microfluidic device design using polystyrene beads in place of cells. Using the final design, beads were able to flow with a steady rate through the trapping area without flowing into the extraction zone. Further, beads were able to be individually trapped and dragged to the extraction zone successfully. It was therefore determined that the device should be tested with human cells.

Semen Samples

Sperm cells were tweezed from diluted semen samples to ensure that the device was functional with human cells. It was noted that during the trapping process, some cells would become stuck to the surface of the coverslip. Over time they would form aggregations and eventually block the channel. Thus, a dilution of 1:20 semen in 4 mg/mL of BSA was chosen as the starting sample concentration to mitigate this issue. With this concentration, the aggregations took a significant amount of time (over one hour) to build and were thus not a serious issue for the trapping process, as sample collection could easily be complete within that window.

Overall, approximately 4.38pg of DNA was obtained from each trapped sperm cell (Table 1). The majority of semen samples possessed higher total yields than theoretical yields (Figure 3, Table 1). This may have been caused by unwanted DNA entering the trapping area, either by random migration or having been stuck to a trapped sperm cell. However, these higher total yields could also partially be explained due to the fact that a more conservative value was used to calculate the expected yield for each sample. When estimating the expected DNA yield, a value of 3.0pg was used as the approximation for how much DNA a single sperm cell contained. Studies have found slightly varying results when determining the amount of DNA in a human sperm cell, ranging from approximately 3.1pg – 3.9pg of DNA per cell [48].

The degradation index (DI) was also analyzed for each sample during quantification. A value under 1 indicates no evidence of degradation to the DNA is present. Only one of the 10 samples had a DI value slightly above 1 (Table 1). There were no significant differences observed in the DI values between samples, with an average DI value of approximately 0.8. This evidence suggests there is no trend of degradation among samples that have been separated using optical trapping, confirming our previous findings [18,19].

STR profiles were generated for each sample and analyzed for completeness, number of drop-in alleles, and average peak height for each locus. It was noted that a sample containing as few as 30 cells was able to produce a profile with over 80% of expected alleles present (Figure 4). Full profiles containing 100% of expected alleles were produced with samples containing as few as 41 cells. On average, approximately 2 drop-in alleles were observed per trapping attempt; only four of nine samples processed contained drop-in alleles (Table 2). Three of these samples containing 2, 6, and 12 drop-in alleles are believed to be caused by analyst error and are not believed to have resulted from the trapping process itself. As discussed previously, extraction zones in early samples were physically pried apart prior to the extraction process. However, this became quite difficult as later pieces became resistant to separating. Attempts were made for some period of time (15-30 minutes) to separate these pieces for the three samples containing 2, 6, and 12 drop-in alleles. It is likely this process led to the contamination and the observation of drop-in alleles seen.

A linear regression was fit comparing the correlation between the number of cells in a sample and the percentage of expected alleles seen in the resulting STR profile (Figure 5, Figure S1). The resulting line had a slope of 0.39457 (95% CI: 0.2353476, 0.5537888), and an R^2 value of 0.8032 which indicated that the number of cells in the sample explained ~80% of the variability in the percentage of expected alleles seen. The global f-test (test statistic = 32.66, df = (1, 8), p-value < 0.001) suggested that the proportion of variability in the percentage of expected alleles explained by the number of cells in the sample was greater than 0 and a positive association between the two variables existed. The Q-Q Plot of the residuals was also visualized and determined to have no departure from normality (Figure 6). This correlation supports that

results are in accordance with the device trapping the number of cells indicated, as it would be expected that a sample containing more cells should produce a fuller STR profile.

Semen and Vaginal Fluid Mixture Samples

Overall, approximately 4.72pg of DNA was obtained from each trapped sperm cell (Table 3). The trend of most samples having higher total yields than theoretical yields was also observed in the mock sexual assault samples (Figure 7, Table 3). It can be argued here, however, that these larger total yields are not a result of DNA randomly flowing into the extraction zone. Very few samples (18%) had any sort of signal above the analytical threshold that could be attributed to the female contributor when considering the resulting STR profiles (Table 4).

Of the fourteen samples collected from mixture samples, four had DI values slightly above 1 suggesting mild degradation was present (Table 3). Of these samples, all but one were relatively close to a value of 1. It is unknown what caused a larger degradation index in one sample. However, given that each cell is trapped for approximately the same amount of time (less than 2 minutes) and that this issue occurred with only one sample, it is unlikely that this can be attributed to the process of optical trapping itself.

A complete STR profile was able to be developed from a sample containing only 31 sperm cells (Figures 8, 9). One of the primary concerns with this device was the potential for unwanted cells and DNA to migrate into the extraction zone. However, on average, only 0.36 non-sperm alleles were observed per trapping attempt (Table 4). Theoretically, only sperm cells were being trapped from the mixture and therefore the presence of the female donor's DNA would alert to this issue. Only four samples out of the total eleven (36% of mixtures tested) had drop-in alleles that could not be attributed to the female donor, with each sample only containing

a single drop-in allele. Two mixture samples (18% of mixtures tested) contained alleles that could potentially be attributed to the female donor (Table 4). These samples contained 31 and 41 cells and had one and three alleles that could be attributed to the female donor, respectively.

In order to assess the sterility of the microfluidic device, substrate controls were also collected alongside samples and analyzed. DNA was not detected during quantification and no STR signal was observed.

Similarly to the neat semen samples, a linear regression was fit comparing the correlation between the number of cells in each sample collected from mock sexual assault samples and the percentage of expected alleles seen in the resulting STR profile (Figure 10). The slope was estimated to be 0.34466 (95% CI: 0.1965857, 0.4927385) with an R^2 value of 0.7047. This indicated that the number of cells in the sample explained ~70% of the variability in the percentage of expected alleles seen. The global f-test (test statistic = 26.25, df = (1, 11), p-value < 0.001) suggested that the proportion of variability in the percentage of expected alleles explained by the number of cells in the sample was greater than 0 and a positive association between the two variables existed. The Q-Q Plot of the residuals was determined to have no obvious departure from normality (Figure 11). Overall, this correlation again supports that the results are in accordance with the device trapping the number of cells indicated.

Collection Speed

The amount of time required to prepare the device for trapping was approximately 5-10 minutes, depending on how quickly the water initially flowed through the device. The amount of time required to trap each sample was recorded ranged from 0.93 to 2.3 minutes per cell for sample collection. Neat semen samples required an average of 1.37 minutes per cell for sample

collection (Table 5), while mixture samples required an average of 1.67 minutes per cell for sample collection (Table 6). Much of the variation seen between sample type, number of cells, and time taken can be attributed to the fact that this is a human-operated technique and has a resulting learning curve.

Conclusions

Overall, cells were able to successfully flow through the newly designed optimized microfluidic device in a manner that allowed trapping to occur, and trapped cells could be effectively isolated and removed from the remainder of the sample. The excised microdevice piece was successfully incorporated directly into the extraction process in a manner that would not disrupt a typical forensic laboratory workflow.

Complete STR profiles were developed from neat semen samples containing as few as 41 cells. In mock sexual assault samples, complete STR profiles were developed from as few as 31 sperm cells. Furthermore, only 36% of mock sexual assault samples contained any drop-in alleles, and only 18% contained alleles that could possibly have originated from the female donor.

Sample preparation for this technique was very minimal, only requiring reconstitution of swabs in sterile water. The longest sample collection took approximately 1.5 hours with most samples averaging under an hour to collect. This technique is generally able to quickly produce samples, especially when considering the lack of differential steps required during extraction and ease of analyzing resulting STR profiles. However, before addressing any further sample sets using this method, more work must be done to refine the techniques used at each specific step of the workflow. For example, the series of cuts used to fully separate the extraction zone from the

remainder of the device could occasionally cause the device pieces to split apart as noted previously. In order to reduce the amount of variability in the final results that originates from sources other than the number of separated cells, further research must be performed to completely solidify the process.

Future projects using this technique should include an expanded sample set to further validate the results seen in this study. In addition, samples that more accurately represent mock sexual assault evidence such as resuspended mixture samples and degraded samples should be used to determine the full viability of this device for casework purposes. It may also be possible to explore the use of computer software to control the laser and microscope to trap cells from the mixture, thereby lessening the amount of work required for the analyst and creating a more automated system. Holographic trapping should also be considered as it would allow multiple traps to be maneuvered simultaneously, thereby increasing the number of cells that could be trapped at once [49]. It has been noted in previous studies that epithelial cells are harder to trap because of their size, but if a stronger laser was utilized, it would be beneficial to modify the device to include multiple extraction areas where cells of each type could be placed in different extraction areas for subsequent analysis [18,19]. If some of these avenues are explored, this microfluidic device could aid in the ability to use optical trapping for casework for crime laboratories. The format explored in this study would allow sperm cells to be completely isolated from a cell mixture sample within a reasonable timeframe and result in single-source profiles that would alleviate the issue of analyzing mixtures.

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Figures & Tables

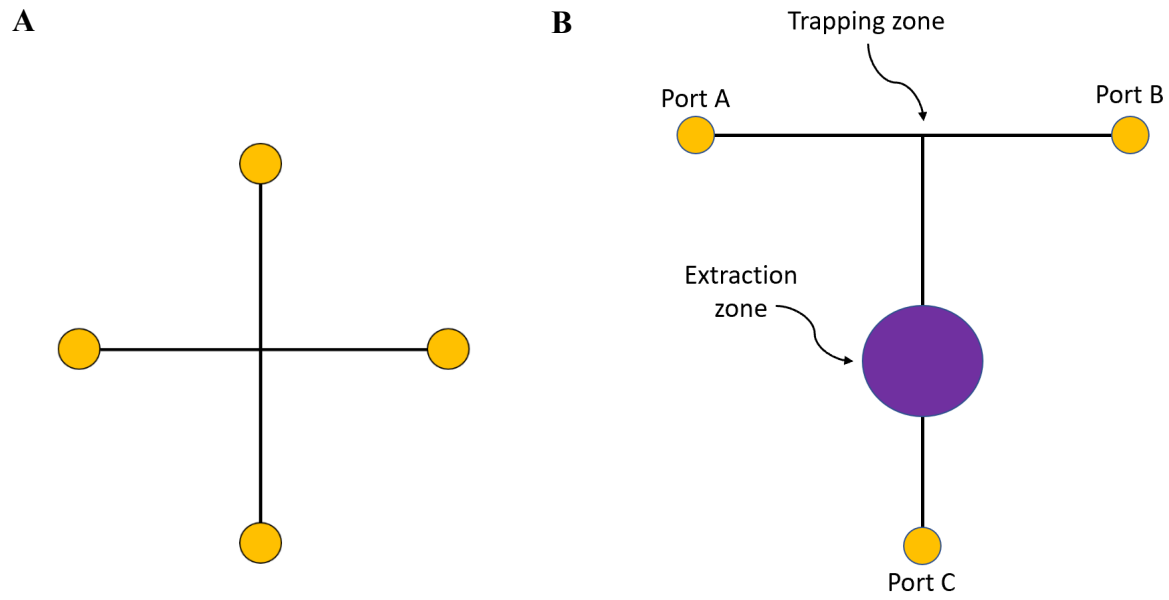

Figure 1: Initial and final designs of the microfluidic device.

A) Initial design of the microfluidic device where open ports are represented in gold and the channels are represented in black, B) Diagram of final chip design where open ports are represented in gold, channels are represented in black, and arrows are indicating the trapping and extraction zones.

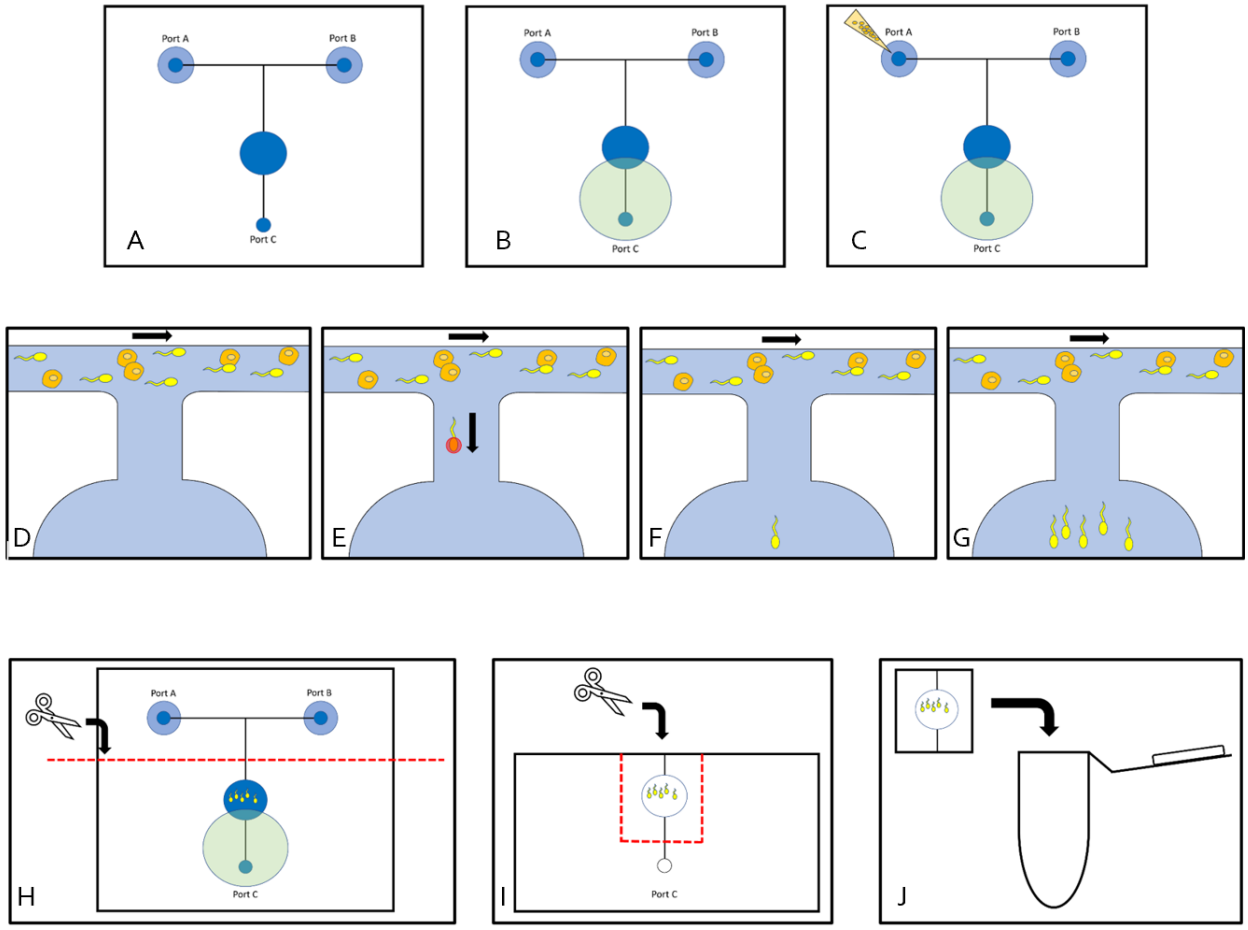

Figure 2: Diagram detailing how the microfluidic device is utilized for the process of cell separation via optical trapping.

A) the device is first filled with ddH_2O so that water forms pockets on the surface at ports A and B, B) Kwik-Cast™ is used to seal port C, C) sample is pipetted into the surface pocket of water at either port A or port B, D) cells begin flowing through the device, E) individual sperm cells are trapped from the trapping area and dragged towards the extraction zone, F) sperm cells are deposited in the extraction zone , G) this process is repeated until the target number of cells have been captured, H) the device is physically cut so that the extraction zone is separated from the cell mixture flow, I) the extraction zone is allowed time to dry and is then cut from the remainder of the device, J) the piece containing the extraction zone is placed into a 2.0 mL tube which is then stored at -20°C until extraction.

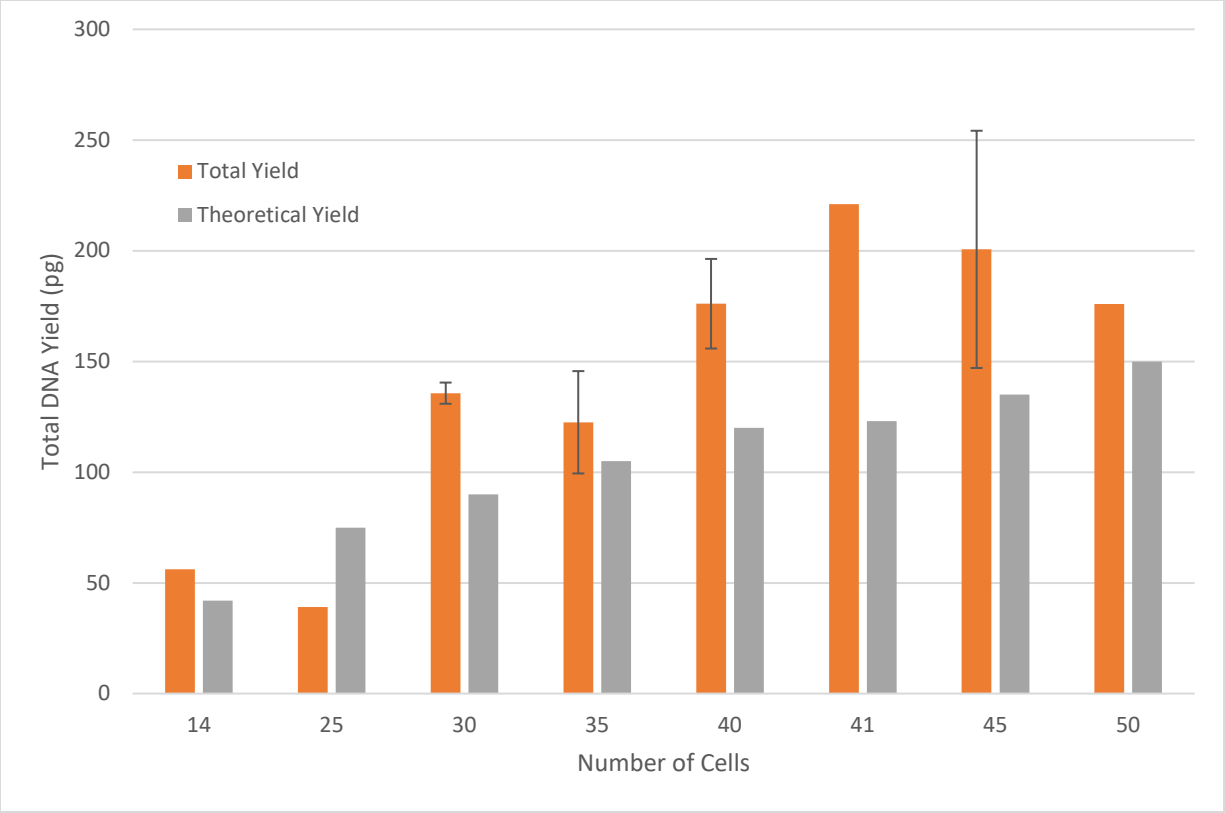

Figure 3: Comparison of total and theoretical yields for samples collected from diluted 1:20 semen.

Error bars indicate there are multiple samples with that cell number.

Table 1: Quantification results from trapping of diluted 1:20 semen samples

Number of Cells	Total Yield (pg)	Theoretical Yield (pg)	Degradation Index (DI)
14	56.1	42	0.95
25	39.1	75	0.84
30	130.9	90	0.73
30	140.5	90	0.58
35	98.4	105	0.49
35	144.7	105	0.72
35	124.5	105	0.99
40	196.3	120	1.27
40	155.8	120	0.96
41	221.1	123	0.63
45	254.2	135	0.81
45	147.1	135	0.89
50	175.9	150	0.89
Average Per Cell	4.38	3	-

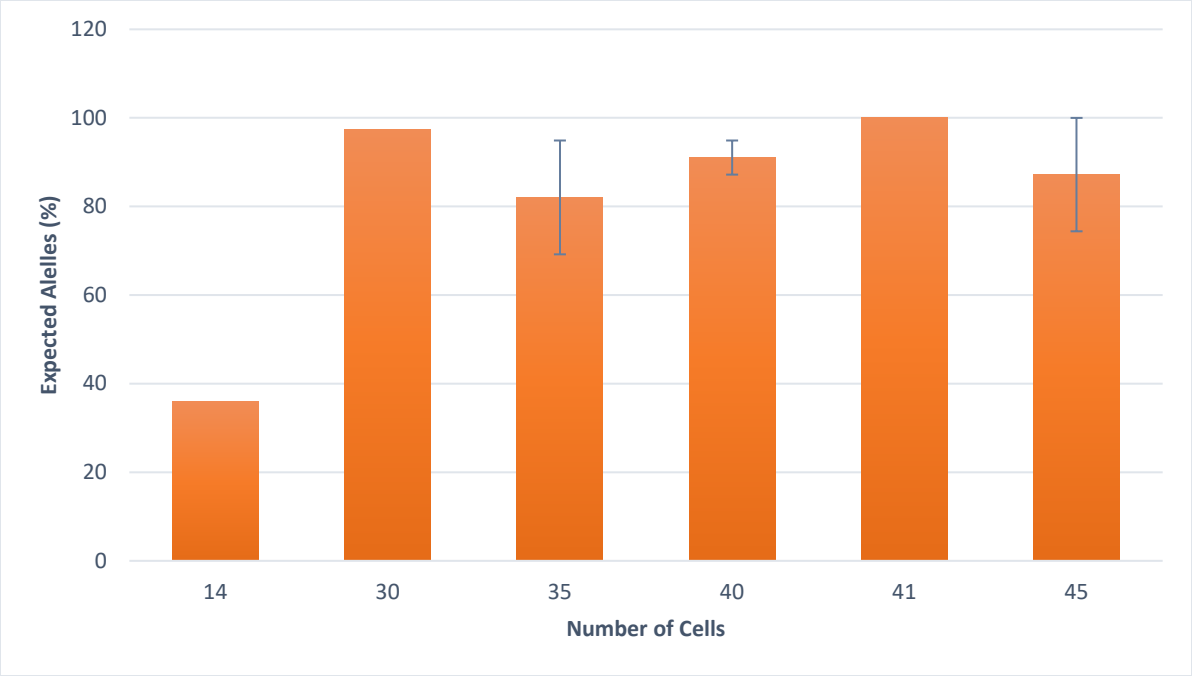

Figure 4: Percentage of expected alleles observed in the resulting STR profiles for samples collected from diluted 1:20 semen.

Error bars indicate there are multiple samples at that cell number.

Table 2: STR profile results from trapping of diluted 1:20 semen samples

Number of Cells	Expected Alleles (%)	Drop-In Alleles	Average Peak Height (RFU)
14	35.9	0	165.53 ± 46
30	97.4	6	347.27 ± 243
35	69.2	0	134.03 ± 59
35	94.9	0	237.93 ± 124
40	87.2	2	136.77 ± 87
40	94.9	0	209.69 ± 94
41	100	0	749.38 ± 275
45	74.4	12	138.84 ± 71
45	100	2	244.16 ± 104
Average	-	2.44	-

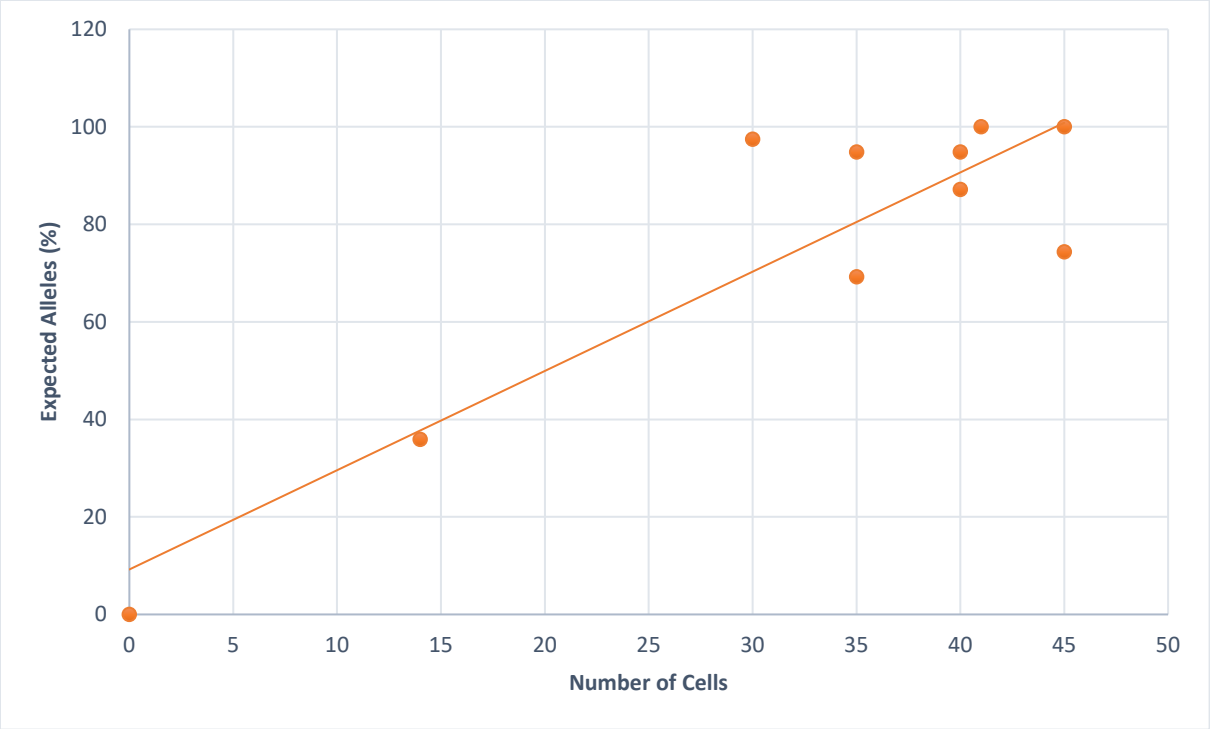

Figure 5: Linear regression fit comparing neat semen sample cell numbers and percentages of expected alleles.

Linear regression fit for neat samples between the number of cells in each sample and the percentage of expected alleles observed in the resulting STR profiles. $R^2 = 0.8032$, p-value < 0.001.

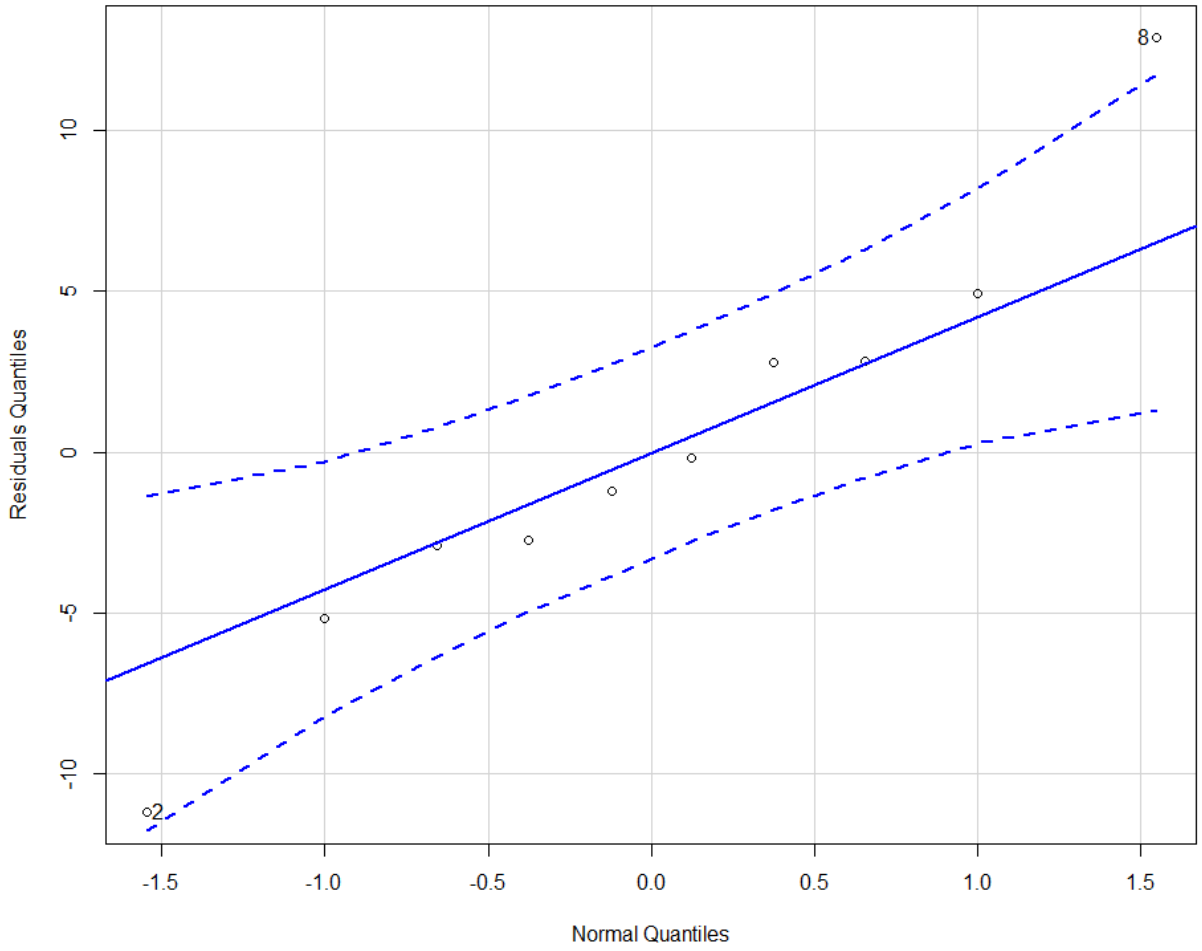

Figure 6: Q-Q plot of residuals from the neat semen sample linear regression.

Q-Q plot assessing the normality of the residuals for the linear regression fit for neat semen samples between the number of cells in each sample and the percentage of expected alleles seen in the resulting STR profile.

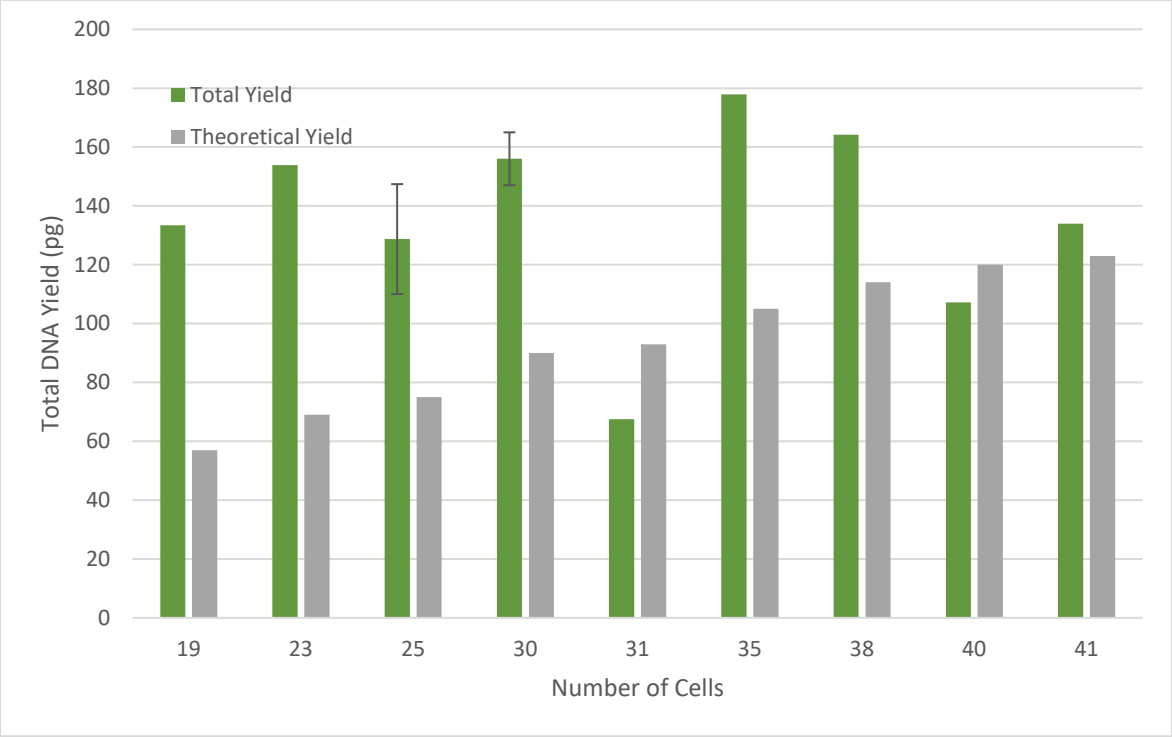

Figure 7: Comparison of total and theoretical yields for mock sexual assault samples.

Error bars indicate there are multiple samples at that cell number.

Table 3: Quantification results from trapping of mock sexual assault samples

Number of Cells	Total Yield (pg)	Theoretical Yield (pg)	Degradation Index (DI)
19	133.4	57	0.61
23	153.8	69	0.73
25	147.4	75	0.75
25	110.0	75	0.87
30	165.0	90	3.14
30	147.0	90	0.69
31	67.5	93	0.31
35	177.8	105	1.39
38	164.2	114	1.29
40	107.2	120	1.44
41	133.9	123	0.83
Average Per Cell	4.72	3	-

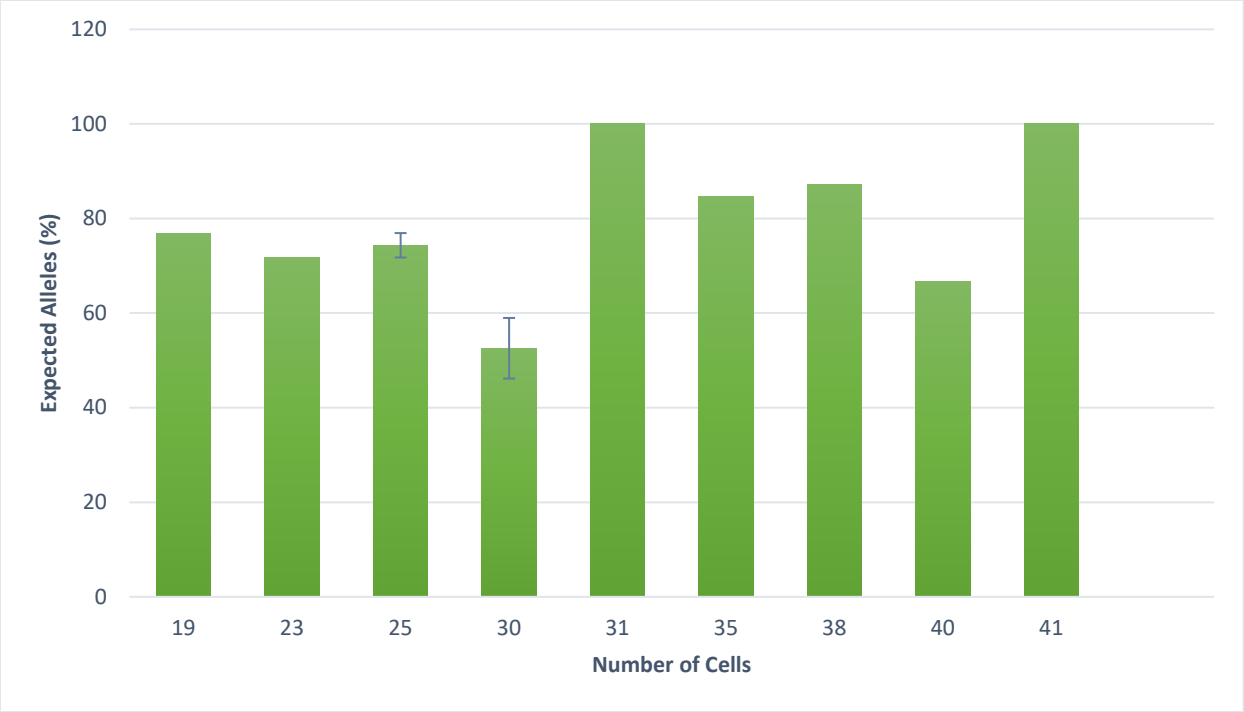

Figure 8: Percentage of expected alleles observed in the resulting STR profiles for mock sexual assault samples.

Error bars indicate there are multiple samples at that cell number.

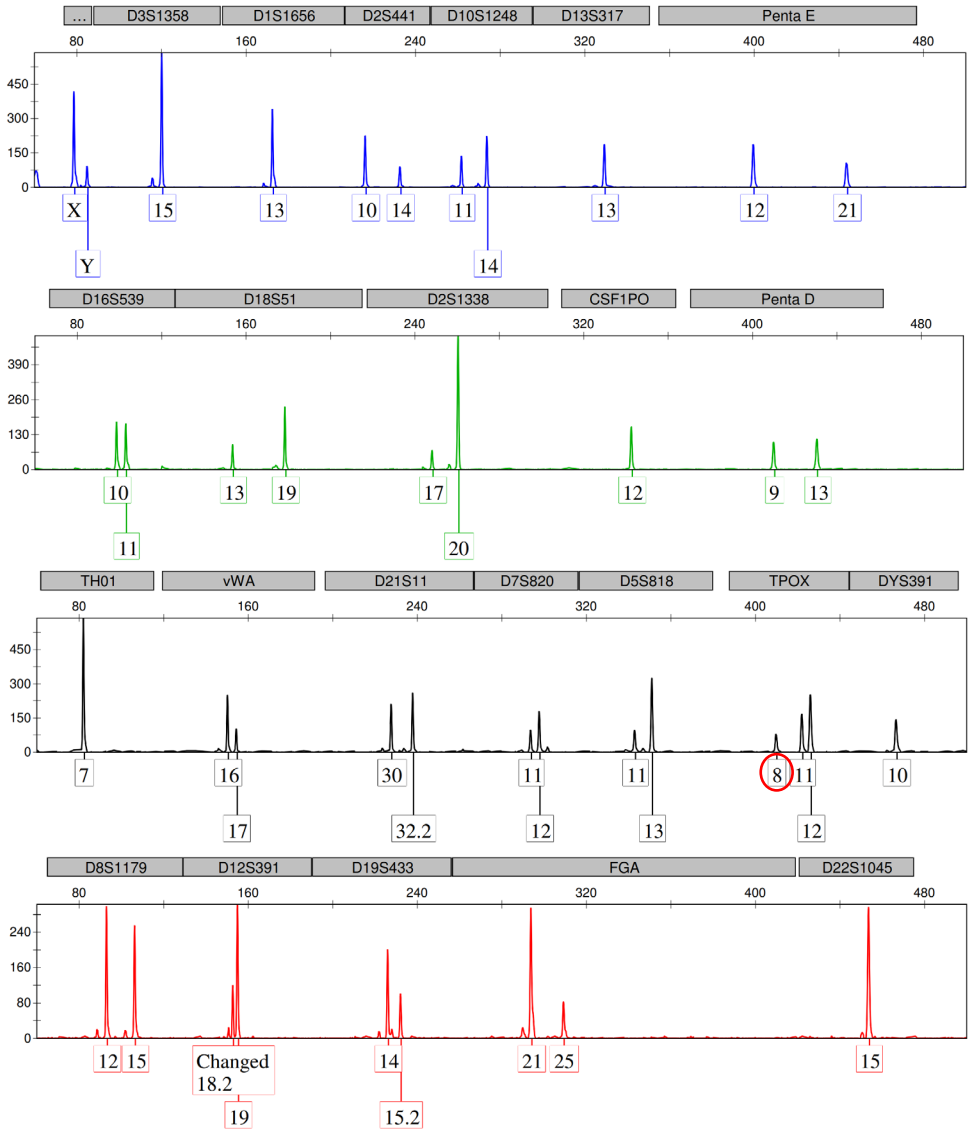

Figure 9: Complete STR profile resulting from the mock sexual assault sample containing 31 cells.

One allele potentially originating from the female contributor is seen at the TPOX locus (circled in red).

Table 4: STR profile results from trapping of mock sexual assault samples

Number of Cells	Expected Alleles (%)	Drop-In Alleles	Female Contributor	Average Peak Height (RFU)
19	76.9	0	0	130.51 ± 98
23	71.8	0	0	103.50 ± 51
25	76.9	1	0	126.94 ± 80
25	71.8	0	0	101.79 ± 50
30	46.2	0	0	83.44 ± 33
30	58.9	1	0	113.33 ± 56
31	100	0	1	184.39 ± 96
35	84.6	0	0	142.46 ± 70
38	87.2	1	0	152.88 ± 76
40	66.7	1	0	120.06 ± 75
41	100	0	3	271.21 ± 117
Average	-	0.36	0.36	-

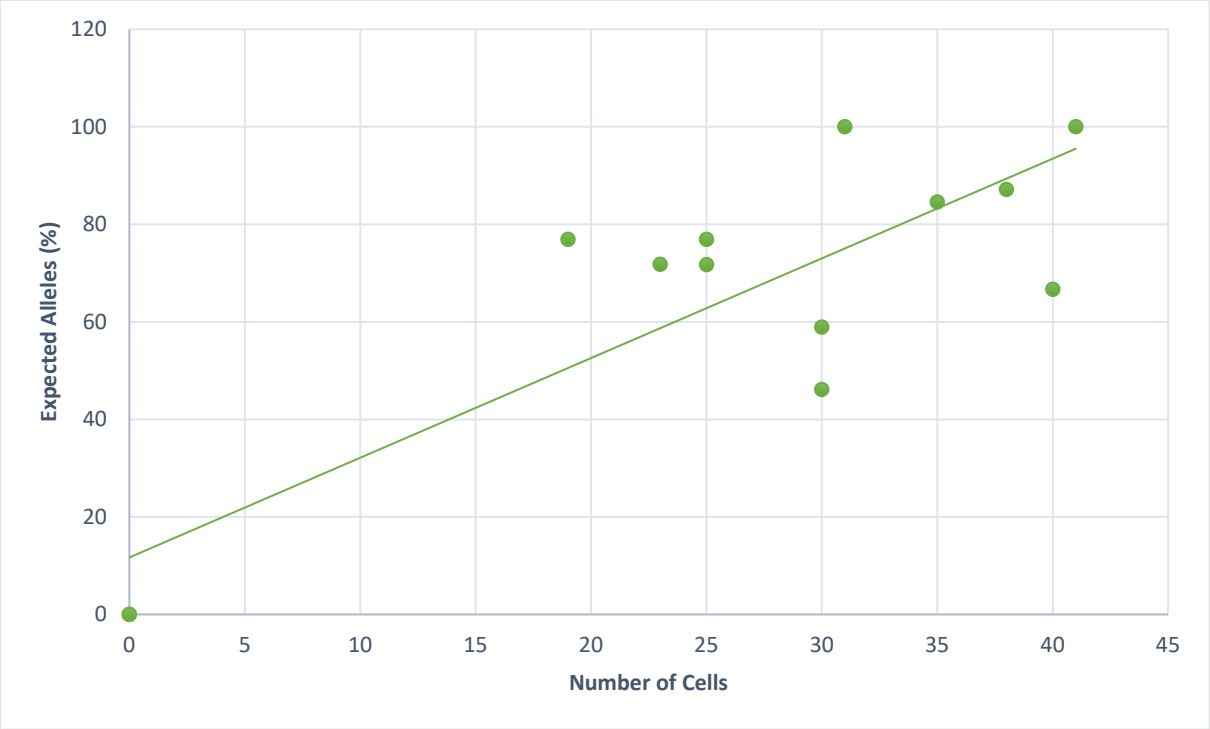

Figure 10: Linear regression fit comparing mock sexual assault sample cell numbers and percentages of expected alleles.

Linear regression fit for mock sexual assault samples between the number of cells in each sample and the percentage of expected alleles observed in the resulting STR profiles. $R^2 = 0.7047$, p-value < 0.001.

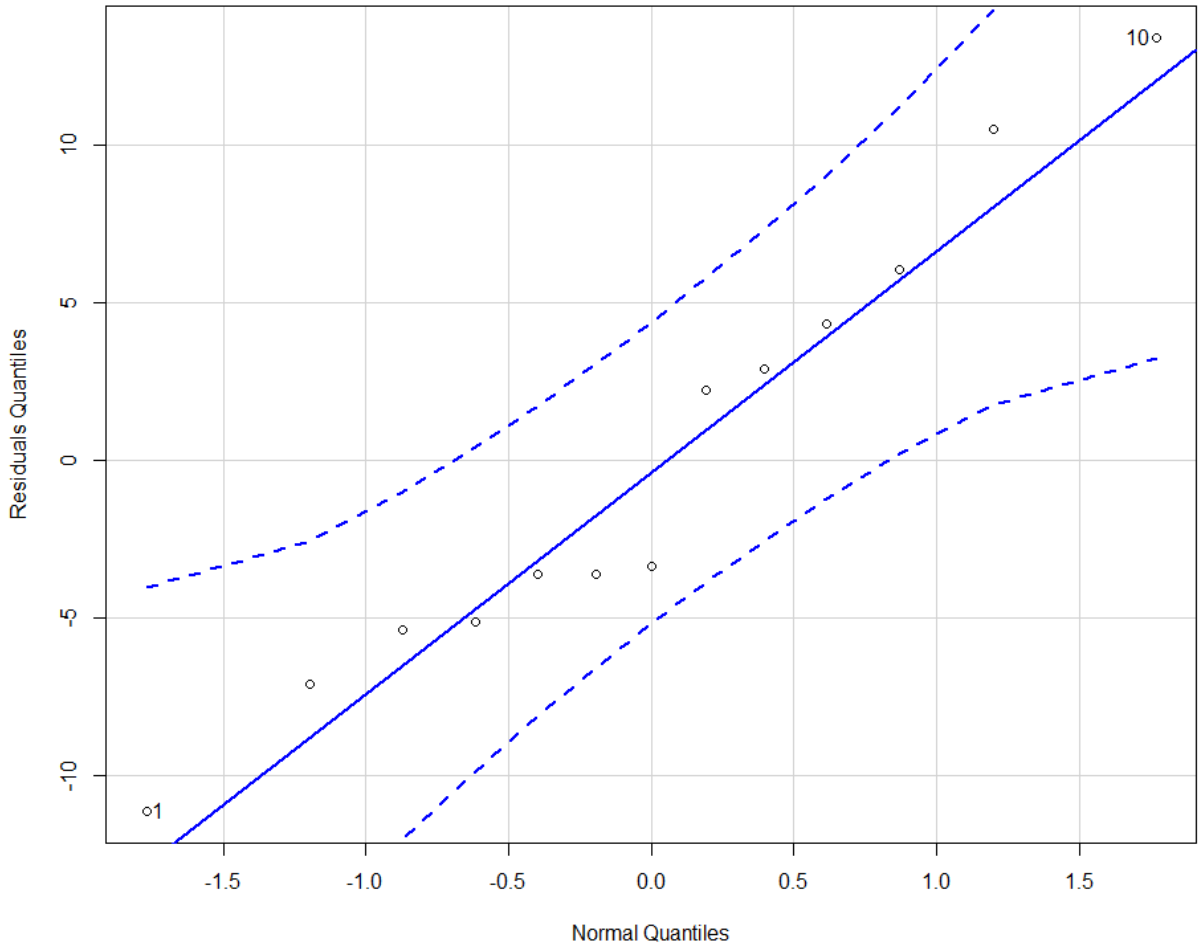

Figure 11: Q-Q plot of residuals from the mock sexual assault sample linear regression.

Q-Q plot assessing the normality of the residuals for the linear regression fit for mock sexual assault samples between the number of cells in each sample and the percentage of expected alleles seen in the resulting STR profile.

Table 5: Amount of time taken to trap cells from neat semen samples

Number of Cells	Time (minutes)	Time (minutes) per Cell
24	38	1.58
30	52	1.73
30	28	0.93
35	45	1.29
35	39	1.11
35	38	1.09
40	50	1.25
40	48	1.2
41	96	2.34
44	72	1.63
45	49	1.09
45	48	1.07
50	79	1.58
Total Average	53	1.37

Table 6: Amount of time taken to trap cells from mock sexual assault samples

Number of Cells	Time (minutes)	Time (minutes) Per Cell
19	40	2.11
20	27	1.35
23	33	1.43
25	45	1.8
25	42	1.68
30	44	1.46
30	60	2
31	64	2.06
35	59	1.69
38	58	1.53
40	73	1.83
40	66	1.65
41	48	1.17
Total Average	51	1.67

Supplemental Figures

To estimate simple linear regression:

```
reg_1_dataset <- lm(data=dataset,variable1~variable2)

confint(reg_1_dataset,level=0.95)
```

To create Q-Q Plots to check assumptions from linear regressions:

```
qqPlot(reg_1_dataset$residuals,
        main="QQ Plot of Residuals",
        xlab="Normal Quantiles",
         ylab="Residuals Quantiles")
```

Figure S1: R code used to calculate a simple linear regression and assess the normality of residuals between cell number and percentage of expected alleles for neat semen and mock sexual assault samples.

Vita

Mackenzie Lally was born in Greenville, SC in 1996. She graduated from Clemson University in 2019 with a B.S. in Genetics and minors in Psychology and Biological Sciences. During her time at Clemson University she was involved with research projects investigating the expression levels of neuroplasticity genes in sailfin molly fish, as well as comparing neural crest cell migration differences in species of cichlid fishes. She joined the M.S. in Forensic Science program at Virginia Commonwealth University in 2019 and investigated the use of optical trapping as a cell mixture separation technique in the Williams laboratory. During her time at VCU, she served as a lead graduate teaching assistant for the forensic science department where she assisted in teaching both undergraduate and graduate level forensic serology and DNA analysis laboratory courses.